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ABSTRACT:

Background: *Plasmodium falciparum* is transmitted from humans to *Anopheles* mosquito vectors via the sexual erythrocytic forms termed gametocytes. Erythrocyte filtration through microsphere layers (microsphiltration) had shown that circulating gametocytes were deformable. Compounds reducing gametocyte deformability would induce their splenic clearance, thus remove them from the blood circulation and block malaria transmission.

Methods: The hand-made, single-sample prototype for microsphiltration was miniaturized to a 96-well microtitre plate format, and gametocyte retention in the microsphere filters quantified by high-content imaging. The stiffening activity of 40 pharmacological compounds was assessed in microtitre plates, using a small molecule (calyculin) as a positive control. The stiffening activity of calyculin was assessed in spleen-mimetic microfluidic chips and in macrophage-depleted mice.

Results: Marked mechanical retention (80-90%) of mature gametocytes was obtained in microplates following exposure to calyculin at concentrations with no effect on parasite viability. Of the 40 compounds tested, including 20 anti-malarials, only 5 endoperoxides significantly increased gametocyte retention (1.5–2.5 fold; 24 hour-exposure at 1 µM). Mature gametocytes exposed to calyculin accumulated in microfluidic chips, and were cleared from the circulation of macrophage-depleted mice as rapidly as heat-stiffened erythrocytes, thus confirming results obtained using the microsphiltration assay.

Conclusions: An automated miniaturized approach to select compounds for their gametocyte-stiffening effect has been established. Stiffening induces gametocyte clearance both *in vitro* and *in vivo*. Based on physiologically validated tools, this screening cascade can identify novel compounds and uncover new targets to block malaria transmission. Innovative applications in hematology are also envisioned.

Key words: malaria; *Plasmodium falciparum*, transmission, deformability, spleen, clearance, screening, microspheres, gametocytes, high content imaging.

INTRODUCTION:

Every 2-3 hours, human red blood cells (RBC) enter the open and slow microcirculation of the spleen where they cross 2 µm-wide inter-endothelial slits before returning to the general circulation [1]. In several inherited or acquired diseases, RBC are unable to deform sufficiently to overcome this mechanical challenge [2]. Their splenic retention subsequently induces anemia and splenomegaly [3]. *Plasmodium falciparum* (*Pf*)-malaria is the most frequently acquired disease where RBC deformability is affected [4]. Intra-erythrocytic asexual parasitic stages develop during a 2-day replication cycle, where rings evolve to multi-nucleated schizonts that rupture the host RBC, thus releasing daughter merozoites which re-invade new RBC [4]. Asexual maturation results in extensive RBC remodeling [5] through parasite growth and exported proteins which restructure the RBC and trigger stiffening [6]. Rings are modestly deformable, a proportion of them circulate [7]. Mature-asexual stages are rigid and sequester in micro-vessels where they are protected from splenic mechanical clearance [8]. *Pf* transmission from humans to mosquitoes relies on the conversion of a small fraction of asexual parasites to sexual stages, called gametocytes [9]. *P. falciparum* gametocytes develop throughout a 2-week period during which immature stages are sequestered, predominantly in the extravascular compartment of the bone marrow [10, 11]. Remodeling of the erythrocyte and parasite membrane also occurs during sexual development [12, 13]. Stiff, immature gametocytes become deformable mature gametocytes that are released into the peripheral circulation [14, 15]. Circulating mature gametocytes must then repeatedly cross splenic inter-endothelial slits. Their ability to traverse these slits is essential for their persistence in circulation and availability to mosquitoes [1].

One strategy towards *Pf*-malaria elimination focuses on the discovery of compounds blocking gametocyte transmission from humans to mosquitoes. Several screening assays have been

reported [1, 16-24] which identified small molecules that kill gametocytes or affect their development in the mosquito. To expand the repertoire of transmission-blocking compounds and uncover new modes of action, we propose a unique screening approach to identify compounds that will stiffen mature gametocytes, thus triggering their splenic mechanical retention [25]. Once cleared from the circulation, mature gametocytes will be removed from the transmission cycle.

Most existing methods to study the deformability of *Pf*-infected RBC [7, 26, 27] are not yet compatible with the stringent requirements for high throughput screening (HTS). A single-sample microsphere filtration (microsphiltration) device to measure the ability of RBC to squeeze between calibrated microspheres, thus mimicking the spleen, has been recently developed [28]. The physiological relevance of microsphiltration was demonstrated by obtaining similar retention rates for abnormally deformable RBC in the device and in human spleens perfused *ex-vivo* [28]. We report here on the development and validation of a microplate version of the microsphiltration device. Microplate filtration is well adapted to screen for compounds that will stiffen mature gametocytes. Pharmacological stiffening observed in the microsphiltration assay translated into gametocyte entrapment in microfluidic chips and in their clearance from the circulation of macrophage-depleted mice. Based on these physiologically validated tools, this approach can identify novel compounds and uncover new targets to block malaria transmission.

MATERIALS AND METHODS:

Preparation of microsphiltering microplates

Calibrated microsphere mixtures (AMTech® 96.5% tin, 3% silver, 0.5% copper), 25-45µm and 5-15µm were resuspended at 2 and 3g/mL, respectively, in phosphate buffered saline (PBS, Sigma) supplemented with 0.5% Albumax II (Life Technologies), then allowed to settle. Fifty µL of 25-45µm microsphere solution was transferred to 96-deep well filter bottom plate (Harvard Apparatus) using a Biomek 3000 liquid handler (Beckman Coulter), then 60µL of 5-15µm microspheres were added, resulting in an ~1.15mm-thick layer.

Parasite culture

The *Plasmodium falciparum*, NF54-derived Pfs16-luciferase-GFP transgenic clone 3 [29], was cultured as described [16, 30] using RPMI 1640 with L-Glutamine and 25mM 4-(2-hydroxyethyl)-1-piperazine-ethane-sulfonic acid (HEPES) supplemented with 2.5mg/ml Albumax II (Life Technologies), 5% AB+ Male Human Serum, 50µg/mL of Hypoxanthine and 2µg/ml Blasticidin (Sigma). Gametocyte growth media without blasticidin was supplemented with 0.5M N-acetyl-glucosamine (NAG) and 11mM Glucose (Sigma). Highly synchronous gametocyte cultures were induced from asexual cultures [16] within a 12 hour window at the outset of gametocytogenesis and uniformly differentiated Stage V gametocytes were harvested at day 12 or 13 post-induction for deformability experiments. For selected mouse experiments, mixtures of immature and mature gametocytes were used.

Light microscopy

Thin blood films were fixed in 100% methanol, stained with Giemsa (Sigma) and counted using 100X oil immersion objective and bright-field illumination. The percentage sexual or asexual stages was counted against 10^4 RBC.

PKH26 and PKH67-labeling of normal and heated RBC

RBC were washed with PBS and heated at 50°C for 20 minutes. Heated and control RBC were labeled using PKH26 or PKH67 Fluorescent Cell Linker Kit for General Cell Membrane Labeling (Sigma Aldrich) as described [28]. PKH-labeled RBC were resuspended in assay buffer (for asexual) or PBS-Albumax II 1% and mixed 1:20 with unlabeled RBC.

Flow cytometry

PKH-labeled RBC were quantified with a BD Accuri C6 flow cytometer using 488nm (PKH-67) and 640nm (PKH-26) laser excitation. Cells were resuspended in PBS at 10^6/mL and parasitemia determined from acquisition of >30x10^3 events. Data were collected and processed using the BD Accuri C6 software.

Microsphiltration

Following loading with stage V mature gametocyte (200µL per well at 2% hematocrit, 5% gametocytemia) or control samples, microtitre plates were vacuum aspirated using a manifold system (Beckman Coulter) coupled to an electric High Output vacuum pump (Millipore) via a 10L trap. Sample aspiration (peak pressure of 7 In.Hg) was followed by a rinsing step with 1.6mL of gametocyte culture medium without NAG (vacuum aspiration, peak pressure of 10 In.Hg). The downstream sample of 1.8mL was retrieved and RBC populations quantified.

Compound Testing

Forty-compounds consisting of 20 FDA-approved drugs and 20 antimalarial agents were selected for assay validation. Two hundred µL of mature gametocyte culture at day 11 post-induction, 2.5% hematocrit and ~3-5% parasitemia were dispensed into polypropylene plates containing compounds at 5µM or 0.5µM in 50µL of gametocyte growth media and final 0.4% dimethyl sulfoxide concentration (DMSO), then incubated with gas permeable sealed

membranes (Corning) for 24h in a 37°C incubator. As negative and positive control, gametocytes were exposed to 50nM Calyculin, a phosphatase inhibitor without effect on the deformability of uninfected RBC [31] or 0.4% DMSO, respectively.

Opera high content imaging

Following microsphiltration, 4.5µL (approximately 10^5 RBC) of each filtered sample and their corresponding unfiltered control diluted to a final hematocrit of 0.2% were transferred using an Agilent Bravo liquid handler to 384-well CellCarrier imaging plates (Perkin Elmer) containing 45.5µL of Cell Mask Plasma Membrane Orange® stain (Life Technology) diluted 1:13,333 in PBS.

Quantification of gametocytemia was determined using high content confocal imaging (Opera™, Perkin Elmer) through acquisition of 17 images per well using a 20X water immersion objective (NA 0.7) resulting in at least 10^4 erythrocytes per well. GFP-positive gametocytes were detected using 488nm excitation with 520/35nm emission and CellMask Orange labelled erythrocytes were detected with 560nm excitation and 600/40 emission.

Data analysis

Images were processed using the Opera™ Harmony® software (PerkinElmer) using image segmentation algorithms. Gametocytes were classified and counted based on elongation index, size and GFP intensity. RBC were detected using CellMask Orange staining intensity, circularity and size. Gametocytemia (up- and downstream) was defined as the total number of gametocytes detected divided by that of erythrocytes, and the corresponding retention rate was calculated as described [28]. Significance probability for compound-induced gametocyte

stiffening was calculated using non-parametric, U-statistic Mann Whitney test with two tailed P value and confidence interval of 99%.

Microfluidics

The microfluidic biochips were cast in polydimethylsiloxane (PDMS, Sylgard, France), a silicone elastomer produced as described [32]. Each biochip comprised 8 parallel filtering units connected to infusion tubing. Biochips were coated with PBS 1% albumin before all experiments. Cultures containing 5% mature gametocytes at 2% hematocrit were exposed to Calyculin (50 nM) or control DMSO 0.4% for 2 hours prior labeling with Hoechst or Sybr Green (Life Technologies), mixing in a 1:1 ratio and centrifugation. RBC samples were infused using a push-syringe as 100% RBC concentrate of 20µL eluted with PBS-Albumax II 1% under a 200 (+/- 50) µL/hour flow rate and 150 (+/- 50) mbar pressure. Enrichment of mature gametocytes in the slits of each filtering units was quantified from fluorescent microscope images (Leica DMI3000 microscope, using Leica DFC310FX camera controlled LAS Superposition Image software, Leica Micro-système, Nanterre, France) acquired 15 minutes post-infusion. Counting was performed by 3 independent unbiased evaluators.

Splenic mechanical retention of gametocytes in mice

All in vivo experiments were performed in accordance with the protocols of the Pasteur Institute (Paris) and the Guidelines for the Care and Use of Laboratory Animals [33]. Experiments were performed with C57BL6 adult mice (8-12 weeks) injected or not with 100µL clodronate liposome (ClodronateLiposomes.com®, Netherlands) for macrophage depletion 24 and 48 hours prior to the transfusion of human cells. Mice were injected retro-orbitally with 100µL human RBC suspensions at 20% hematocrit (in culture media) containing 40% of heat-stiffened RBC (HRBC) or immature (3-10 days post-induction) or

mature (12-14 days post-induction) *P. falciparum* gametocytes (NF54 or Pf16). For assessment of calyculin stiffening activity, mature gametocyte cultures were exposed for 2 hours to either calyculin (100nM) or control DMSO (0.4%) before injection in clodronated mice. For control experiments, HRBC (50°C, 20 minutes) or control unheated RBC (nRBC) were differentially labeled with PKH-26 and PKH-67, respectively, prior to injection. Blood samples were then collected from the tail vein 1, 90 and 180 minutes post-injection. Circulating concentrations of heated and unheated human RBC were determined by flow cytometry. The concentration of mature and immature gametocytes was determined by microscopic examination of Giemsa-stained blood films.

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RESULTS:

96-well microsphiltration mimics splenic retention of *Pf* infected and abnormal RBC.

To improve throughput, the tip-based microsphiltration method [28] was adapted to a 96-well microplate format. Deep-well plates designed for vacuum-based filtration were prepared with two layers of microspheres, designed to replicate the filtering properties of the tip-based approach. Optimal dispensing of microspheres into the plates was standardized using an automated liquid dispenser (Fig 1-A, b). A two-step vacuum-based aspiration protocol was validated (Figure 1-B). The microsphere matrix remained wet between filtration steps, and the morphology of filtered RBC was preserved (Fig 1D).

Mimicry of splenic mechanical sensing was confirmed by quantifying the retention rates of uninfected RBC and *Pf* asexual blood stages. As expected, weak retention (<10%) was observed for normal RBC (Figure 1-C). Heat-stiffened RBC [35] were retained by more than 90%. As previously observed with the tip-based prototype, ring stage *Pf*-infected RBC showed moderate retention at 35.8 ± 10.7%, while mature asexual stages were retained at 81.5 ± 5.1% (Fig 1-C). The relatively wide distribution of retention rates for rings was related to their increasing loss in surface area to volume ratio as the parasite matures [35,36] and the distribution of ages across different experiments. These retention rates represent a dynamic range of filtering capability consistent with previously published results [28] using the single-sample prototype or *ex vivo* human spleen perfusion [37]. These data illustrate the successful development of microplate-based microsphiltration for rapid and robust determination of spleen-like retention of RBC samples.

Increased throughput and automated quantification of gametocytes

Retention rates of RBC harbouring asexual *Pf* stages in the microsphiltration assay were determined using Sybr green and flow cytometry analysis. Retention rates calculated either from Giemsa-stained blood films or flow cytometry showed a strong linear correlation ($r^2 \geq 0.9$; Fig 2-A). Retention rates of gametocytes was determined using high content imaging based on RBC labelling with a fluorescent membrane stain and gametocyte GFP expression [29]. A strong linear correlation ($r^2 = 0.96$) was obtained between manual counting using a hemocytometer and imaging-based quantification, across the full range of mature gametocyte concentrations relevant for this assay (Figure 2B).

Proof of concept for pharmacological screening using microsphiltering microplates

Using the microsphiltration assay, untreated or DMSO-exposed, mature gametocytes exhibited less than 50% retention (Figure 3-A). Exposure to 50nM calyculin for 2 and 24 hours resulted in a significant increase in retention to 89.85 ± 5.17% and 79.26 ± 3.21% ($p < 0.0001$), respectively. Evaluation of calyculin gametocytocidal activity using a mitotracker-based viability assay [17] showed a <25% inhibition at 100nM (Fig 3-A). The difference in retention rates between DMSO and calyculin-exposed gametocytes provided a screening window stable across 3 independent biological replicates, with an average Z' score of 0.67.

Forty compounds, including 20 anti-malarial agents and 20 FDA-approved drugs were tested using the assay. None of the 20 FDA-approved drugs significantly increased gametocyte retention above negative control values. Of the 20 anti-malarial agents tested with a 24-hour exposure, only 5 endoperoxide derivatives, namely artemether, dihydro-artemisimin (DHA), artesunate (ART), artemisinin and artemisone were found active at 1μM ($p < 0.0001$) (Fig 3-B, C, D). Assessment of gametocytocidal activity for these compounds revealed killing activity at concentrations lower than those inducing mechanical retention (Supplementary Fig

S1). The microtitre plate-based microsphiltration platform thus determined the gametocyte-stiffening effect of multiple compounds in parallel.

Physiological relevance of the screening process

To confirm that a compound identified as active in the microsphiltration assay induces the mechanical blockade of compound-exposed gametocytes in the spleen, we analyzed the fate of gametocytes treated with calyculin *in vitro* and *in vivo*.

Mechanical blockade in a spleen-mimetic microfluidic chip: Mature gametocytes from 2 different laboratory strains and a sample directly collected from the blood of a *P. falciparum*-infected-patient were exposed to calyculin at 50 nM for 2 hours, then infused through microfluidic chips. This method has been shown to recapitulate the mechanical constraints imposed on RBC as they cross inter-endothelial slits in the human spleen and validated with an array of poorly deformable RBC [32]. Exposure to calyculin induced a marked enrichment to 72.40 ± 13.64% in mature gametocytes in the narrowest slits of the chips versus 27.60 ± 13.64% for the DMSO-exposed gametocytes (Fig 4A) ($p < 0.0001$). Thus, the altered mechanical behavior of pharmacologically-stiffened mature gametocytes observed with the microsphiltration assay was confirmed using microfluidic chips as an independent measure of spleen-like retention. This provides a physiologically relevant validation of active compounds identified by microsphiltration.

Clearance of pharmacologically-stiffened gametocytes from the blood of macrophage-depleted mice: Gametocytes exposed to either calyculin or 0.4% DMSO were transfused into C56Bl6 mice previously treated with clodronate liposomes to deplete endogenous macrophages and allow normal human RBC to circulate for several hours (Fig 4B, C and D). As expected, when untreated mice were transfused with human RBC a complete clearance

was observed in less than 90 minutes. By contrast, 73.19 ± 26.87% and 58.53 ± 12.97% of human RBC ($p = 0.0006$) were still in circulation at 90 and 180 minutes, respectively, after transfusion into macrophage-depleted mice. In these macrophage-depleted mice, only 35.63 ± 24.42% and 22.98 ± 6.94% of stiff, heat treated RBC remained in circulation after 90 and 180 minutes, corresponding to >75% clearance, arguably by a macrophage-independent, mechanical process (Fig 4B). When gametocyte circulation kinetics were examined, we found that 81.21% of immature compared to 15.35% of mature gametocytes were cleared after 90 minutes ($p = 0,0005$). This result is consistent with previous studies which demonstrated a marked difference in the stiffness of immature and mature stages using biorheological methods [14, 15] (Fig 4C). Finally, mature gametocytes exposed to calyculin for 2 hours had a clearance rate of 77.52%, whereas exposure to 0.4% DMSO resulted in only 34.83% retention after 180 minutes ($p = 0.0286$). Interestingly, these clearance rate kinetics were almost identical with those of nRBC (26.97%) and HRBC (77.01%). Hence, a compound active in the microsphiltration assay induces the splenic mechanical retention of gametocytes in vivo (Fig 4D).

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Discussion:

Using a miniaturized microsphiltration assay designed to mimic the mechanical sensing of RBC by the spleen, we have demonstrated that a small molecule can significantly impair the ability of mature *P. falciparum* gametocytes to cross spleen-like slits, independently from gametocyte killing. This finding was confirmed *in vitro* using biomimetic microfluidic chips, and *in vivo* using a macrophage-depleted mouse model. Collectively, these results establish an original screening and post-screening cascade to select compounds that induce the mechanical retention of mature gametocytes in the human spleen, thus eliminating them from the malaria transmission cycle. Once retained in the spleen, the fate of a RBC is macrophage-mediated phagocytosis and destruction. Mechanical retention of RBC is effective in malaria patients [1] [34]. Therefore, compounds which induce retention and clearance of mature gametocytes in the spleen represent solid candidates as transmission-blocking agents.

To find transmission-blocking agents through an original, biomechanical approach we optimized the manual microsphiltration prototype to a miniaturized automated format. This effectively streamlined the processing of 96 samples in a simultaneous fashion with the potential to screen up to 5,000-10,000 compounds in single point, over a 1 to 2-month period. We have also developed specific tools to validate this approach. Lavazec and colleagues recently identified compounds interfering with a signalling pathway that regulates gametocyte-infected RBC deformability. The identification of calyculin [31] has provided the much needed tool for validation of the screening approach reported here. We have demonstrated that calyculin stiffens gametocytes at concentrations lower than those affecting gametocyte viability (Fig 3). This stiffening activity was further confirmed using biomimetic microchips with narrow 2 micron-wide slits [32] in which gametocytes exposed to calyculin accumulated after a few minutes of infusion (Fig 4A). To validate the effect of hits in vivo,

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we developed a new transfusion approach in mice. The mouse and human spleens retain autologous spherocytic RBC at a similar pace [38, 34] and the minimal surface area loss triggering biomechanical retention is also similar in both species, namely >18% in human spleens [35] and >20% in mice [38]. Macrophage depletion was used to allow human RBC to remain in the peripheral circulation of C57BL6 mice. As expected, those human RBC were rapidly cleared from the circulation of macrophage-intact controls (Fig 4B), while the majority of them were still in the peripheral blood of macrophage-depleted mice 3 hours after xenotransfusion. In this model, a minority of mature gametocytes and a majority of immature gametocytes were cleared, consistent with previous observations *in vitro* [14]. Similarly, only a minority of mature gametocytes exposed to DMSO and a majority of those exposed to calyculin were cleared. This strongly suggests that calyculin induces the clearance of mature gametocytes in vivo by inducing their mechanical retention in the spleen [33, 40].

In the first set of compounds tested, most were inactive and induced retention rates very similar to those observed with the solvent control (DMSO) (Fig 3). Five endoperoxides were identified as actives, however, as opposed to calyculin, induced retention only at concentrations higher than those affecting viability (Fig 3 & Fig S1). Mechanical retention was thus more likely a consequence of gametocyte killing rather than a new mode of action of endoperoxides on gametocyte carriage. Interestingly, artemether affected the viability of mature gametocytes at lower concentrations than dihydroartemisinin. This is consistent with the greater effect artemether-lumefantrine has on gametocyte carriage and transmission to Anopheles compared to DHA-piperaquine [41].

The ideal compound selected by our screening/post-screening cascade would induce a potent, irreversible increase in the retention of mature gametocytes and have a long half-life. Duration of gametocyte carriage is reduced from 7 to 2 weeks in malaria patients treated with

329 ACTs [42, 43]. A mean duration of activity of two weeks would thus cover the gametocyte
330 circulation period in ACT-treated patients. What is the minimal activity that would translate
331 into public health benefit? Modeling indicates that the prediction is complex, due to a possibly
332 non-linear relationship between gametocytemia and transmission to Anopheles [44] and a
333 wide diversity of epidemiological situations. While moderately active compounds (eg, 80%
334 retention) would impact transmission from subjects carrying >250-300 gametocytes/µl, only
335 solidly active compounds (> 98% retention) would have an impact in subjects with <20
336 gametocytes/µl. Whatever the anti-gametocyte approach used, reducing transmission from
337 subjects with intermediate gametocytemia (20-200 gametocytes/µl) will require very potent
338 activity. If modeling accurately reflects reality, combining a gametocyte-killing agent from
339 ongoing screening activities [16-24] with a gametocyte-stiffening agent from our
340 biomechanical approach may increase the likelihood for transmission-blocking success.

341 Following assay optimization, microplate-based microsphiltration proved robust and flexible.
342 The results were reproducible across multiple microplate formats, utilizing various automated
343 dispensers, different microsphere sources and microsphiltration media. Optimization by
344 analyzing male/female and reversible/irreversible effects is envisioned. We have developed a
345 storage process that preserves the integrity of the microsphere layer and assay performance
346 for 3 months thereby enabling transport and deferred use at distant laboratories (not shown).
347 Potential applications include HTS to discover compounds modulating the biomechanical
348 properties of RBC in conditions such as diabetes [45], sepsis and inflammatory diseases [46],
349 inherited RBC disorders [47], or transfusion [48]. The assay may also provide a quality
350 control test for RBC concentrates, as poorly deformable subpopulations of RBC tend to
351 accumulate upon storage [28, 49].

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531

FIGURE LEGENDS

Figure 1: From the hand-made, single-sample prototype to a high throughput microphiltration assay using microplates. (A) In the prototype, 5 – 25 µm microspheres were directly deposited by hand above the anti-aerosol filter of a tip. In the microplate assay, 25 to 45µm-diameter microspheres [2] were poured using automated dispenser into 96-well filter mesh plates to support a 1.15 mm-thick layer of 5-15µm microspheres [1] forming the matrix mimicking the geometry of splenic slits [28]. (B) The driving force for microsphiltration in the Tip format was applied using syringes connected to hermetic tubing. The RBC sample was gently pushed by hand into the filter [1]. An electric pump then flushed the rinsing medium across the filter [2]. In the microplate format, microsphiltration was operated by vacuum aspiration in 2 steps; the sample was aspirated into the microsphere layer [1], and then rinsed using microsphiltration medium [2]. The use of deep-well microplates allowed rinsing to be performed in a single step from the filtering to the receiver plate. (C) The microplate format displayed physiologically relevant retention (mean retention ± SD) of an array of RBC subpopulations, including normal (nRBC), heat-stiffened RBC (HRBC), or RBC infected with *P. falciparum* asexual stages (Rings, Troph=Trophozoits, Schiz=Schizonts). (D) Microplate-based microsphiltration is an open system preserving the morphology of infected and uninfected RBC (Giemsa-stained smears up- and down-stream from filters).

Figure 2: Accurate, automated quantification of asexual and sexual stages up- and down-stream from filters. A linear correlation was observed between parasitemia (A) and retention rates (B) determined either by Flow cytometry or by reference method (conventional counting on Giemsa-stained smears under the microscope). Parasite populations were filtered at different stages of asexual maturation under different experimental conditions to cover a

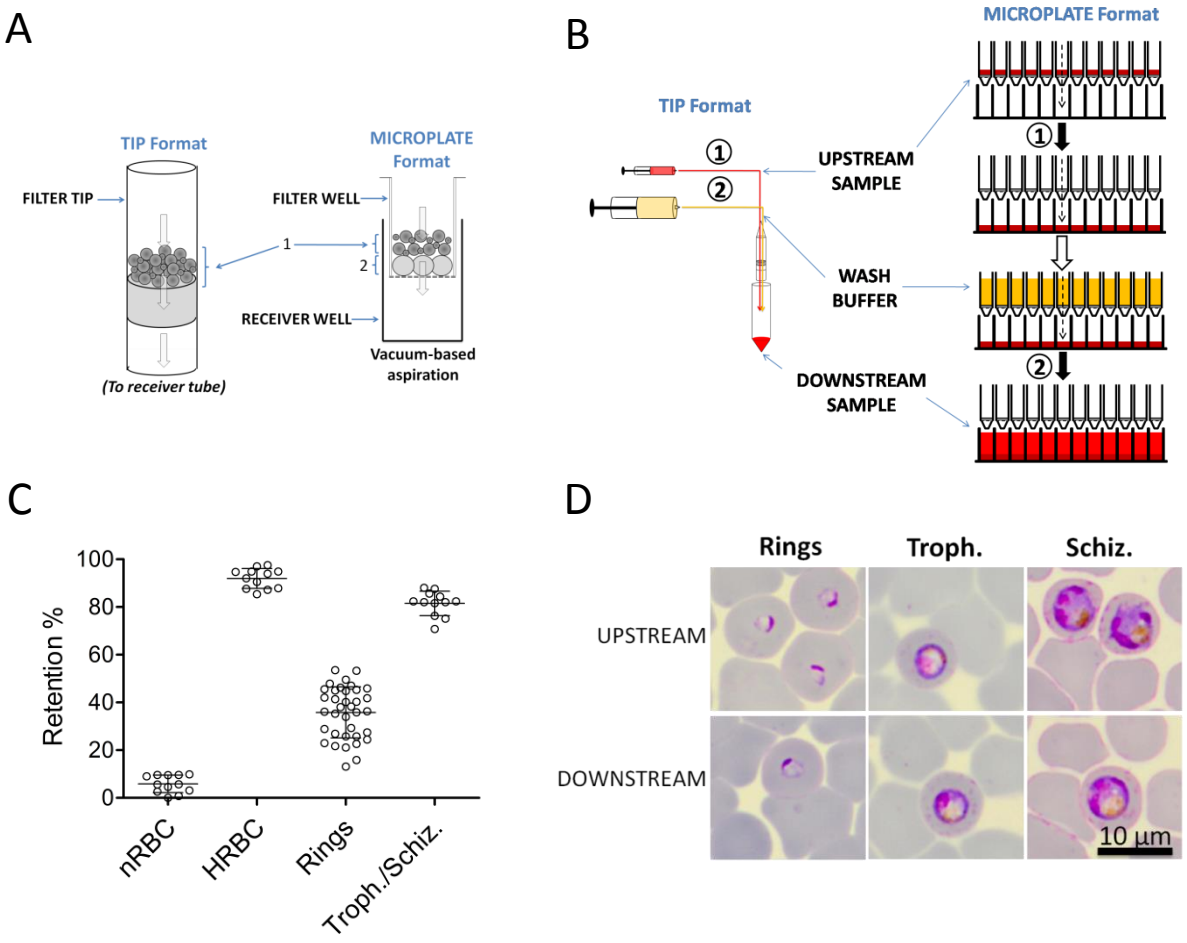
broad range of retention rates. Pooled results from 6 independent experiments are shown. (C) A linear correlation was observed between gametocyte concentrations determined either by Opera high content imaging or by reference method (hemocytometer) (n=3). (D) Example image taken using Opera confocal microscope of GFP-expressing Pf16 stage V gametocytes which were quantified and analyzed automatically.

Figure 3: Robust determination of retention rates after exposure of gametocytes to 40 different compounds as a proof-of-concept for application for screening. (A-i/) Single-well retention rates (mean values ± SD) of mature stage V gametocytes were determined by microplate-based microsphiltration after a 2- or 24-hour exposure to DMSO at 0.4% or calyculin (50 nM). Compared to unexposed controls (GAM V), 0.4% DMSO did not alter gametocyte retention rates (GAM V-DMSO, 2 & 24H) while calyculin significantly increased their mechanical retention (GAM calyculin, 2 & 24H). (A-ii/) The same concentration of calyculin (50 nM) did not significantly affect the viability of mature gametocytes at 24H. (B) Retention rates (mean values ± SEM) of stage V gametocytes measured after a 24H exposure with 40 pharmacological compounds at 0.1 and 1 µM (n=3). Only artemether, dihydro-artemisimin (DHA), artesunate (ART) artemisinin and artemisone (all endoperoxides) significantly decreased gametocyte ability to cross microsphere filters at 1µM (C, D).

Figure 4: Mechanical clearance of compound-exposed mature gametocytes *in vitro* and *in vivo* to validate screening. A) Mechanical retention (mean values ± SD) of mature gametocytes in the filtering units of spleen-like biomimetic microchips as quantified by fluorescence microscopy. Calyculin-stiffened gametocytes were significantly enriched compared to controls. B) C57BL6 mice were treated (Full line) or not (Doted line) with clodronate liposome for macrophage depletion prior to the transfusion of human RBC. Heat-stiffened human RBC (HRBC, Purple line) or control unheated RBC (nRBC, Red line) were

injected retro-orbitally after differential labeling with PKH-26 and PKH-67), for subsequent quantification using flow cytometry. Blood samples were collected at the tail vein 1, 90 and 180 minutes post-injection (4 independent experiments for a total of 15 mice). C) Macrophage-depleted C57BL6 mice were transfused with human RBC infected with either immature (Dotted line, [stage I-IV]) or mature (Full line, [stage V]) gametocytes. The concentration of circulating gametocytes was determined on Giemsa-stained smears showing the persistence in circulation of a majority of RBC infected with mature gametocytes and the clearance of a majority of RBC infected with immature gametocytes (4 independent experiments for a total of 12 mice). D) Macrophage-depleted C57BL6 mice were transfused with human RBC infected with mature *P. falciparum* gametocytes, exposed just before transfusion either to calyculin at 100 nM or control DMSO 0.4% during 2 hours (2 independent experiments for a total of 8 mice). A majority of mature gametocytes exposed to calyculin (Green line) were cleared while a majority of mature gametocytes exposed to the solvent (Blue line) stayed in circulation ($p = 0.0286$). Red dotted line: nRBC and Purple dotted line: HRBC. Results from these mice experiments are expressed as mean values ± SEM.

Antimicrobial Agents and Chemotherapy



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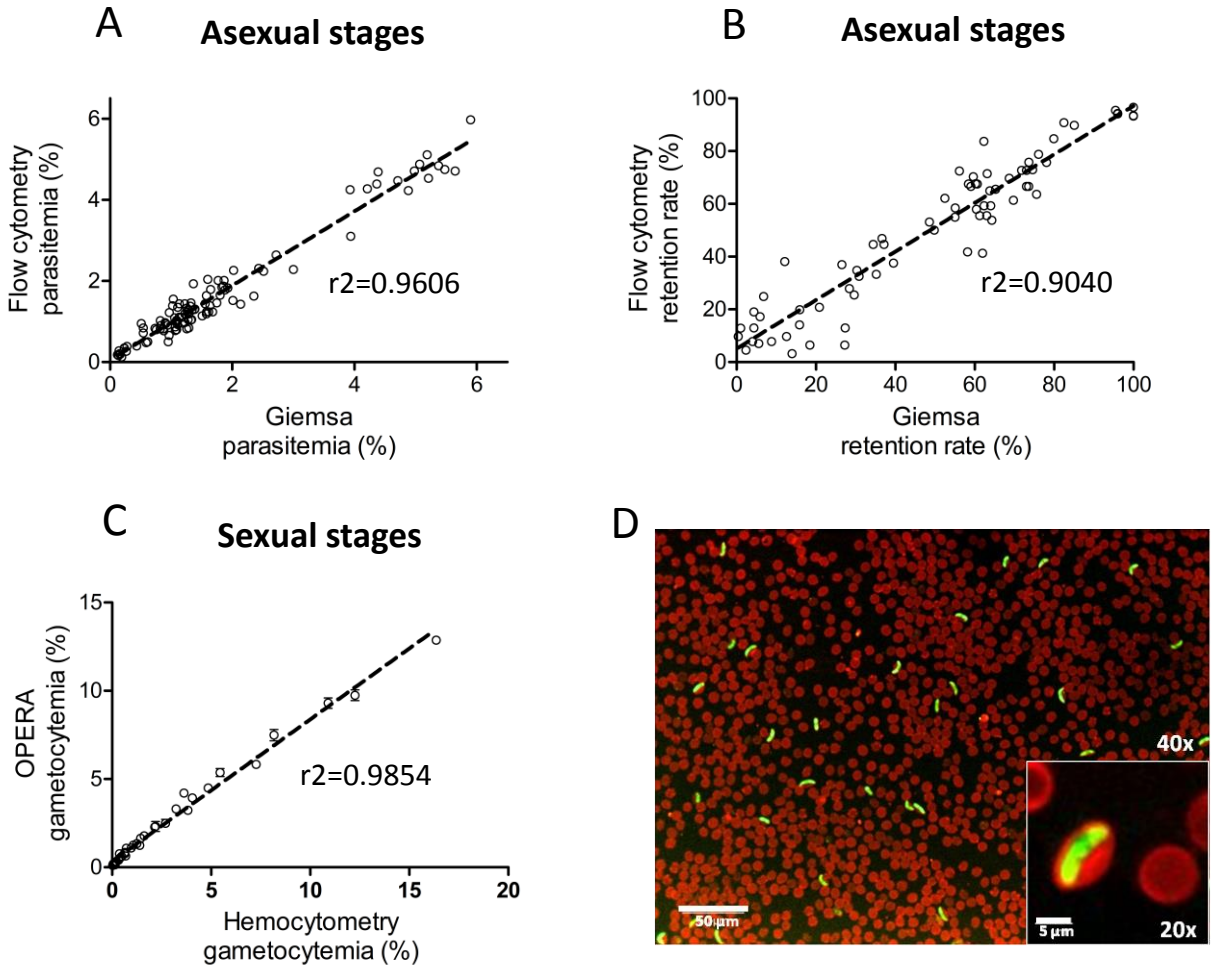

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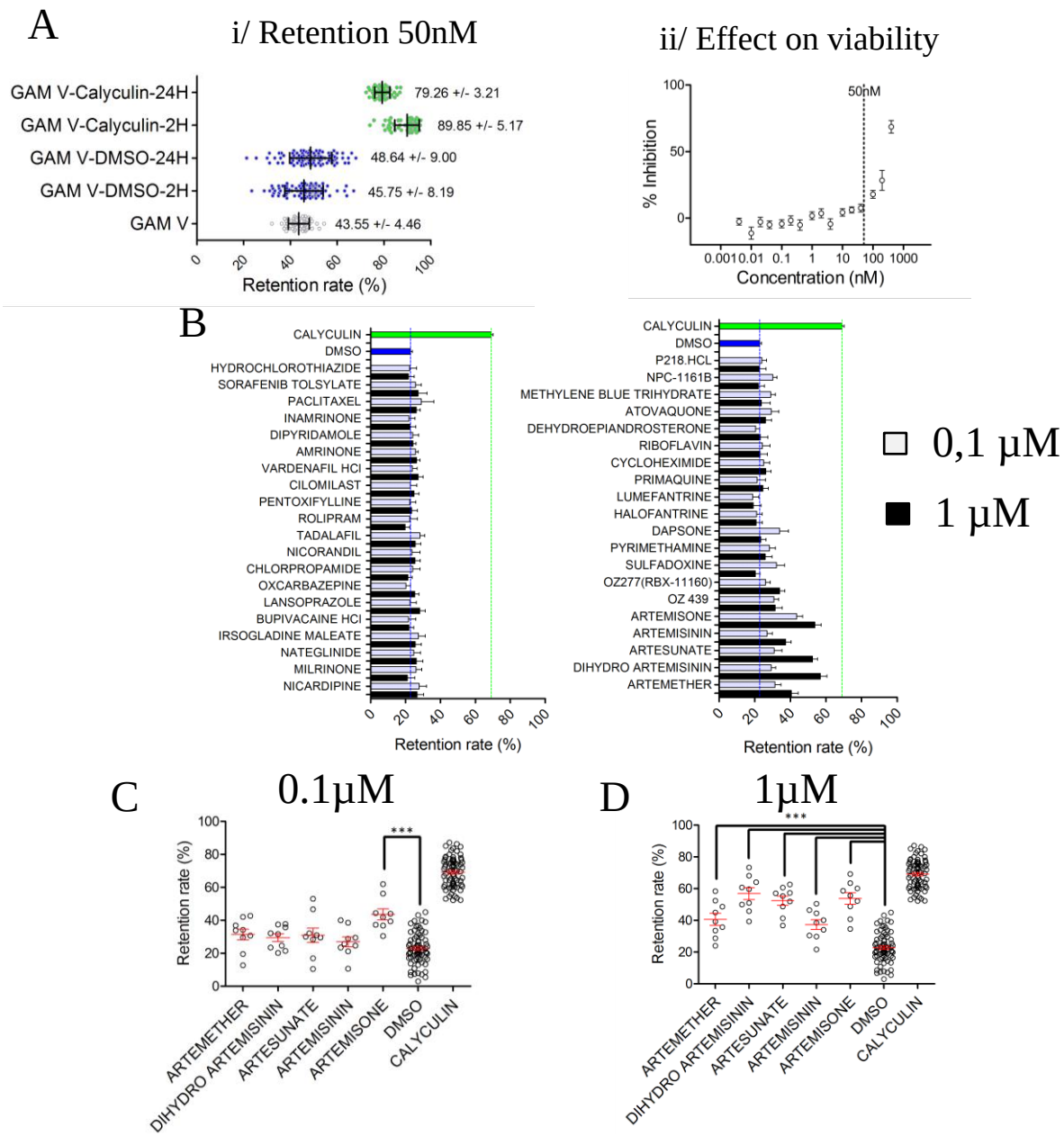

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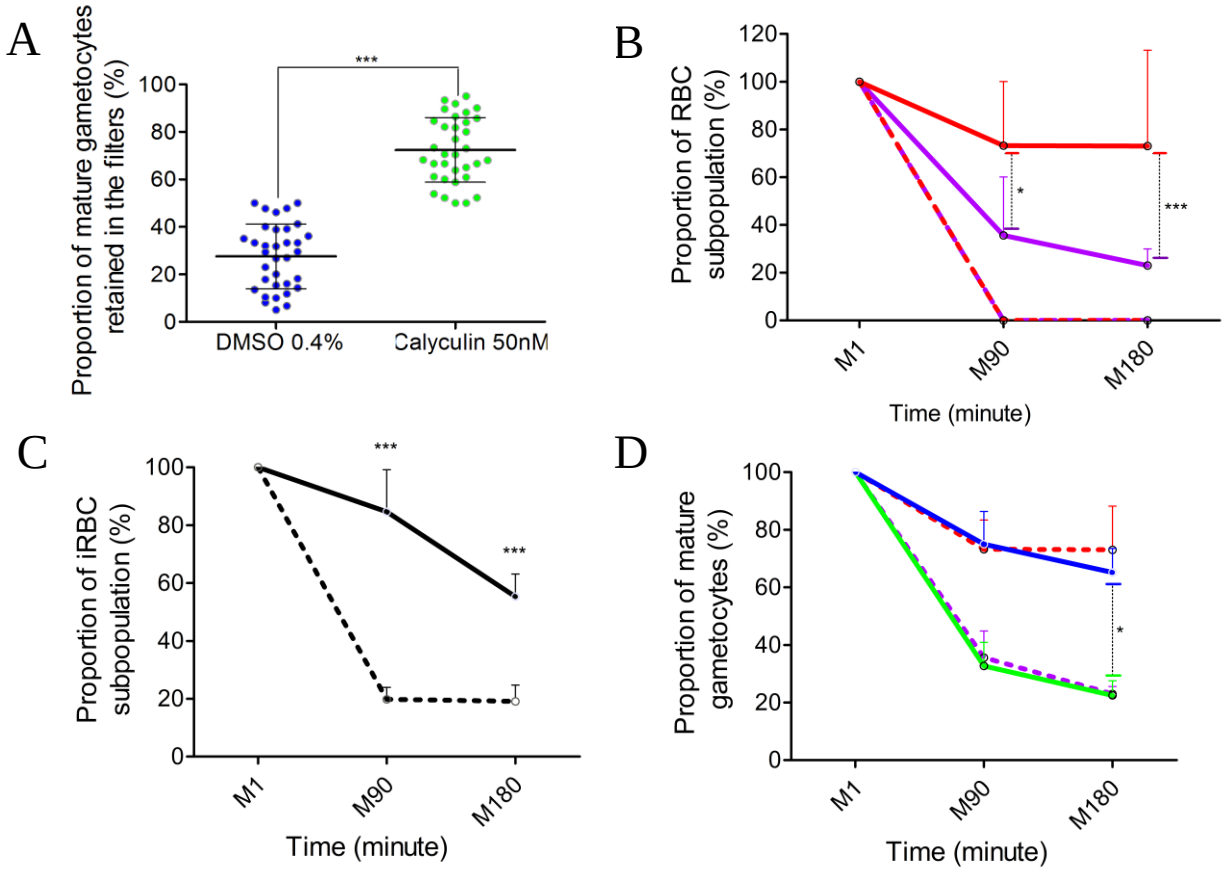

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